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Resolution of Multiple Complexes of Phosphoprotein NS with Nucleocapsid Protein N of Vesicular Stomatitis Virus

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The interaction of the nucleocapsid protein N and the phosphoprotein NS of vesicular stomatitis virus (VSV) was studied, free of other viral proteins, by transcription from SP6 vectors, followed by translation in a rabbit reticulocyte lysate. N-NS complex formation depended strongly on cotranslation of the two proteins; when N and NS were mixed following separate translation of each, very little complex formation occurred. Conditions were found under which at least six N-NS complexes were separated from each other by electrophoresis in a nondenaturing gel system, and the following findings were made. (i) These complexes fell into two groups; complexes 1 through 5 all had a stoichiometry of two molecules of N to one molecule of NS, whereas N-NS complex 6 had an equimolar ratio of the two proteins. (ii) N-NS complexes 1 through 5 predominated at lower concentrations of NS relative to N, but N-NS complex 6 was the major or sole product when NS was equimolar to or in excess of N. (iii) The two sets of complexes were formed by two distinct types of interactions of NS with N. The formation of N-NS complexes 1 through 5 was abolished by the removal of as few as 11 amino acid residues from the basic, highly conserved carboxy-terminal domain of NS, which is essential for the binding of NS to the N-RNA template of VSV. In contrast, formation of complex 6 was unaffected by removal of as many as 62 of the carboxy-terminal amino acids of NS, a region encompassing both the terminal basic domain and an adjacent domain which is required for VSV RNA polymerase function. The significance of these observations for the mechanism of VSV genome replication is discussed.

The nucleocapsid of the rhabdovirus vesicular stomatitis virus (VSV) comprises four components (3). Two of these, the 240-kilodalton large protein L and the 30-kilodalton phosphoprotein NS, make up the RNA-dependent RNA polymerase contained within the virion (12). The other two components, the 11,000-nucleotide negative-strand RNA genome tightly encapsidated by multiple molecules of the nucleocapsid protein N, together constitute the template for the viral polymerase. In the cytoplasm of the infected cell, the VSV nucleocapsid carries out the essential transcription and replication functions of the viral genome. The isolated nucleocapsid very actively transcribes in vitro (2), and in concert with an in vitro protein-synthesizing system or a cell extract source of viral proteins, it can replicate the full-length genome or antigenome (9, 16, 29). This latter requirement is the major distinction between viral transcription and replication. Whereas transcription can be reconstituted from separately purified L, NS, and N-RNA templates (10, 12), replication in vitro requires, additionally, a soluble pool of viral proteins. Similarly, inhibitors of cellular protein synthesis inhibit VSV replication but not transcription in vivo (33).

Elegant in vitro studies have clearly established N as the protein whose synthesis is obligatory for replication (28), and N is thought to act as an antiterminator of RNA synthesis at key points along the genome-length products (6). However, NS also has been shown to play an auxiliary role in that it keeps N in a replication-competent state (17, 30, 31). In addition, NS forms soluble complexes with N both in vivo and in vitro (8, 30, 31).

We have made use of an in vitro system in which N and NS mRNAs were transcribed from SP6 transcription vectors and then translated in a rabbit reticulocyte lysate to study protein-protein and protein-RNA interactions. In this report, we describe the resolution and partial characterization of at least six distinct complexes formed between N and NS. In the following report (26), we describe the binding of nascent N protein to RNA and the effect of NS protein on this binding.

MATERIALS AND METHODS

Plasmid construction. A transcription vector, pN109, linking a full-length copy of the coding region of the N gene of VSV (New Jersey serotype) to the RNA polymerase promoter of bacteriophage SP6 (27) was constructed by standard techniques (22) (Fig. 1). A cDNA clone containing the 5′ portion of the N gene (pN102) was obtained by the method of Gubler and Hoffman (15), by using a deoxyoligonucleotide complementary to nucleotides 643 to 660 of the N mRNA (4) to prime first-strand cDNA synthesis. The construction of pN77, a cDNA clone of the 3′ portion of the N gene, has been described previously (4). The *Ssp*I-*Ssp*I restriction fragment of pN77 was blunt-end ligated into the polylinker region of the SP6 vector pGEM4 (Promega Biotec) following digestion of pGEM4 with *Pst*I and blunting of the resulting 3′ overhang with T4 DNA polymerase in the presence of the four deoxynucleoside triphosphates. An intermediate construct with the correct insert orientation (pN106) was digested at the polylinker *Sac*I site, and after T4 DNA polymerase blunting of the 3′ overhang, the plasmid was then digested with *Pst*I. The isolated large fragment of pN106 thus obtained was ligated to the *Eco*RV-*Pst*I insert fragment isolated from pN102. The resulting plasmid, pN109, when linearized by digestion with *Hin*dIII, encoded an SP6 mRNA

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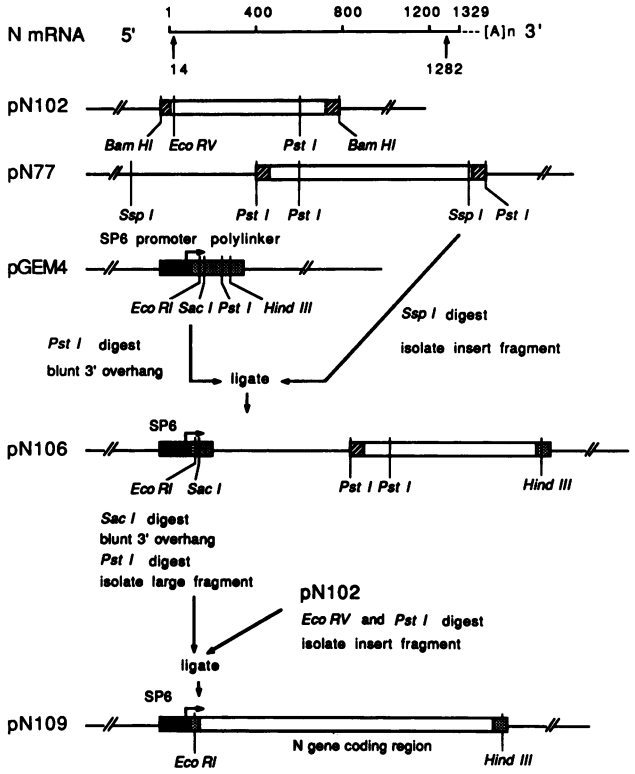

FIG. 1. Construction of pN109, an SP6 transcription vector for the N gene of VSV (New Jersey serotype). The arrows below the line representing N mRNA denote the boundaries of the N protein-coding region. Plasmid pN109 was derived from the linkage of portions of two partial cDNA clones of the N gene (pN102 and pN77) to the bacteriophage SP6 RNA polymerase promoter of pGEM4, as described in Materials and Methods. Plasmids pN102 and pN77 are aligned to show the regions of the N mRNA to which their inserts (open boxes) correspond and the unique internal *Pst*I site which they have in common. The hatched boxes in pN102 and pN77 (not drawn to scale) represent the dC-dG linkers generated during the procedures used to clone these plasmids (4, 15). The SP6 promoter and polylinker regions of pGEM4, pN106, and pN109 (not drawn to scale) are represented by solid and stippled boxes, respectively.

containing nucleotides 8 to 1307 of the authentic N mRNA (4) bounded at the 5′ and 3′ ends by 16 and 8 nucleotides, respectively, of the pGEM4 polylinker sequence. The dideoxy sequencing method of Sanger et al. (32) was used to verify the sequence of the pN102-derived portion of pN109, as well as the sequences across all junctions used in the construction of pN109. Deoxyoligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer. All plasmids were propagated in *Escherichia coli* RR1 or HB101 grown in the presence of 100 μg of ampicillin per ml. Plasmids were purified by two cycles of equilibrium centrifugation in CsCl gradients in the presence of propidium iodide (22).

SP6 transcription reactions. Capped, runoff SP6 mRNAs were synthesized in 200-μl reactions containing 40 mM Tris hydrochloride (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 1 U of RNasin (Promega Biotec) per μl, 500 μM each ATP and CTP, 100 μM each GTP and UTP, 500 μM $m^7GpppGm$ (Pharmacia), 25 μCi of [5,6-^{3}H]UTP (52 Ci/mmol), 4 μg of linearized DNA template, and 40 U of SP6 RNA polymerase. Full-length VSV N and NS transcripts were synthesized from *Hin*dIII-linearized pN109 and *Bam*HI-linearized pGEM-NS2 (14), respectively. Transcription reactions were carried out for 60 min at 40°C. Products were twice extracted with phenol-chloroform and then with chloroform and were twice precipitated with ethanol. The incorporation of labeled UTP was determined by binding to DEAE filter paper (Whatman DE 81); typically, a 200-μl reaction yielded 8 to 15 μg of RNA. The size and homogeneity of RNA products were verified by electrophoresis in 5% polyacrylamide gels containing 8 M urea.

In vitro protein synthesis. Translations of SP6-derived N and NS mRNAs were carried out in an mRNA-dependent protein-synthesizing system prepared from micrococcal nuclease-treated rabbit reticulocyte lysate (Amersham Corp.). In a standard reaction, 0.2 to 0.3 μg of mRNA in 2 μl of H_2O was added to 10 μl of rabbit reticulocyte lysate containing 1.3 U of RNasin and 1.25 μCi of [^{35}S]methionine (>1,000 Ci/mmol); in some cases, where noted, reactions were carried out at two or three times this scale. Incubation was carried out for 90 min at 30°C. As assayed by [^{35}S]methionine incorporation into either hot trichloroacetic acid-precipitable material or bands excised from gels, the translation efficiencies of the SP6 mRNAs were roughly 0.4 and 1.0 μg of protein per μg of RNA for N and NS, respectively, but these values varied from lot to lot of rabbit reticulocyte lysate (see Results).

PAGE. In vitro-synthesized proteins were analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) by using the standard discontinuous system described by Laemmli (21), with the modification that sodium dodecyl sulfate (SDS) was omitted from all gels and buffers and samples were not heated prior to electrophoresis. Optimal resolution was obtained with gels (22-cm length by 1.5-mm thickness) containing 7.5% acrylamide (0.2% methylene bisacrylamide). Reticulocyte lysate samples (4 μl) were diluted with an equal volume of 2× sample buffer (125 mM Tris hydrochloride [pH 6.8], 450 mM β-mercaptoethanol, 25% glycerol, 0.01% bromphenol blue; SDS free) and run at 100 V at ambient temperature until the tracking dye reached the bottom of the gel (ca. 17 h). Under these conditions, there was no detectable heating of the gel during electrophoresis. Gels were fixed in 50% methanol–10% acetic acid and were impregnated with 1 M sodium salicylate prior to drying and fluorography at −70°C.

For electrophoresis in a second dimension, a wide strip (0.6 to 1.0 cm) containing the relevant lane was excised from the unfixed first-dimension gel, and this strip was equilibrated for 30 min in the appropriate sample buffer prior to being sealed on top of the second-dimension stacking gel with 1% agarose. Second-dimension nondenaturing PAGE was carried out as described above; second-dimension SDS-PAGE (10% acrylamide–0.27% methylene bisacrylamide) was carried out with the system described by Laemmli (21). For the quantitation of N-NS protein stoichiometry, spots were located by autoradiography of unenhanced, dried, second-dimension gels, and these spots were excised and solubilized in 2 ml of 30% H_2O_2 for 48 h at 50°C prior to counting in 8 ml of Aquasol (New England Nuclear Corp.).

Radioisotopes and enzymes. [5,6-^{3}H]UTP, L-[^{35}S]methionine, T4 DNA polymerase, and the restriction endonucleases *Alu*I and *Rsa*I were obtained from Amersham. Cloned SP6 RNA polymerase, *Eco*RV, *Hin*dIII, *Pst*I, *Ssp*I, and *Xmn*I were from New England BioLabs, Inc. *Sac*I was obtained from Boehringer Mannheim Biochemicals; T4 DNA ligase was from Bethesda Research Laboratories.

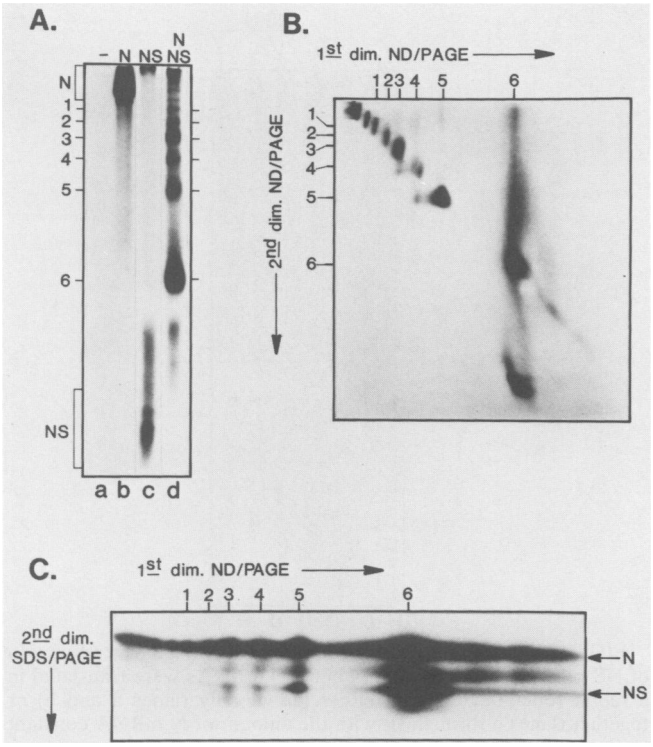

FIG. 2. Resolution of multiple N-NS complexes by nondenaturing PAGE. (A) N and NS mRNAs were translated either individually (lanes b and c) or together (lane d) in a rabbit reticulocyte lysate, and samples were analyzed by nondenaturing PAGE as detailed in Materials and Methods. Lane a contains a control reaction to which no mRNA was added. (B) A sample of cotranslated N and NS proteins was analyzed in two dimensions (dim.), both by nondenaturing (ND) PAGE. Arrows denote the direction of migration in each dimension (− to +). (C) A sample of cotranslated N and NS proteins was analyzed by nondenaturing PAGE in the first dimension and by SDS-PAGE in the second dimension. Arrows denote the direction of migration in each dimension (− to +).

RESULTS

Resolution of multiple N-NS protein complexes by nondenaturing PAGE. To study the interactions between the VSV N and NS proteins in isolation from other viral proteins, N and NS mRNAs were synthesized from SP6 transcription vectors and were translated in a rabbit reticulocyte lysate protein-synthesizing system. Translation products were analyzed by nondenaturing PAGE. In the gel system used, N protein, when translated individually, entered the running gel but remained near the top of the gel as an apparent aggregate of bands (Fig. 2A, lane b). In contrast, NS protein, when translated individually, migrated far into the gel and appeared as a broad streak which sometimes was resolved into three or four diffuse bands (Fig. 2A, lane c). Cotranslation of both N and NS, however, resulted in the disappearance of most of the original N and NS bands and the appearance of at least six new bands at positions intermediate between the original positions of N and NS (Fig. 2A, lane d [bands 1 to 6]. The mobilities of these N-NS protein complexes were 0.15, 0.19, 0.24, 0 31, 0.40, and 0.66 relative to the center of the NS streak. The gap immediately beneath the N-NS complex 6 in this and other nondenaturing PAGE (see Fig. 3 to 5) was found to be due to the exclusion of all other proteins by the relatively large concentration of hemoglobin present in the rabbit reticulocyte lysate; this exclusion was apparent both by the migration of hemin to this locus and from Coomassie blue staining of some gels.

TABLE 1. Stoichiometry of N-NS complexes[a]

Complex	N/NS molar ratio (mean ± SD)	n
1	2.36 ± 0.95	9
2	2.08 ± 0.77	9
3	1.81 ± 0.78	9
4	2.04 ± 1.08	9
5	1.85 ± 0.95	9
6	0.95 ± 0.22	11

[a] The incorporation of [^{35}S]methionine into individual spots in two-dimensional gels similar to that shown in Fig. 2C (first dimension, nondenaturing PAGE; second dimension, SDS PAGE) was determined by excision of spots from gels as described in Materials and Methods. The N and NS proteins each had a significant amount of a very closely migrating secondary band due to limited proteolytic breakdown. For each protein, this secondary band was excised and counted as part of the main band. In a typical set of data, N/NS radioactivity ratios ranged from 4,868:3,084 cpm for complex 1 to 70,420:75,688 cpm for complex 6.

To test whether the observed formation of at least six discrete species of N-NS complexes was somehow an artifact of the buffer conditions of the gel system used, samples identical to those described in the legend to Fig. 2A were analyzed by nondenaturing PAGE with a running gel of pH and ionic strength (120 mM Tris hydrochloride [pH 7.9]) lower than those of the standard buffer system described by Laemmli (375 mM Tris hydrochloride [pH 8.8]) (21). With this gel, we observed the same pattern of bands with the same relative mobilities for N and NS, translated either alone or together, as that seen in Fig. 2A (data not shown). As an additional test, a sample of cotranslated N and NS proteins separated by nondenaturing PAGE (Fig. 2A, lane d) was electrophoresed in a second dimension, also by nondenaturing PAGE (Fig. 2B). With this sample, the separations of most, if not all, of each N-NS complex in both the first and the second dimensions of nondenaturing PAGE were identical, indicating that these bands likely represented discrete species produced in the original translation reaction and were not the result of an equilibrium established under the conditions of gel electrophoresis. It should be noted, however, that in the second dimension there was a small amount of transition of complex 3 to complex 4 and of complex 4 to complex 5. In addition, a significant amount of complex 6 appeared to undergo breakdown or disproportionation into free NS protein and complexes 1 through 5.

The separation of cotranslated N and NS proteins by nondenaturing PAGE, followed by a second dimension of SDS-PAGE (Fig. 2C), established that each of the six putative N-NS complexes did, indeed, contain both N and NS proteins. The resolution of N and NS proteins in the second-dimension SDS-PAGE was complicated somewhat by the fact that each protein ran as a doublet, presumably because of the occurrence of a limited amount of proteolysis during the lengthy separation by nondenaturing PAGE. Nevertheless, by direct determination of radioactivity of protein spots from numerous two-dimensional gels similar to that shown in Fig. 2C, the stoichiometry of N and NS proteins within the various complexes could be calculated on the basis of the known methionine content of each protein (4, 13). It is clear that the molar ratio of N to NS in complex 6 was 1:1 (Table 1). The close migration of this complex to monomer NS indicates that it was composed of a monomer each of N and NS. On the other hand, complexes 1 through 5 all had N/NS molar ratios of 2:1. Thus, these complexes

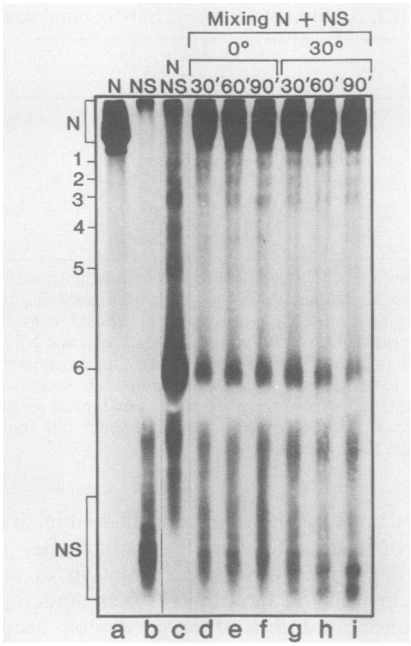

FIG. 3. Comparison of cotranslation versus mixing of N and NS proteins. N and NS mRNAs were translated either individually (lanes a and b) or together (lane c) in a rabbit reticulocyte lysate for the standard 90-min incubation period. N and NS proteins were then mixed after being translated individually and analyzed by nondenaturing PAGE following incubation for 30, 60, or 90 min at either 0 or 30°C (lanes d through i).

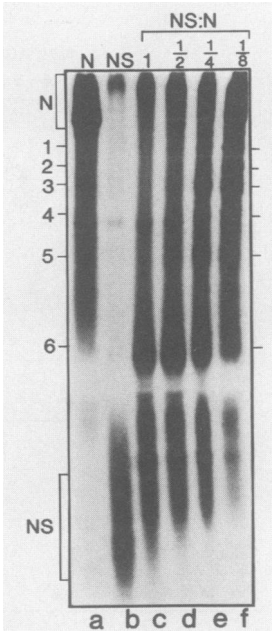

FIG. 4. Formation of N-NS complexes 1 through 6 as a function of NS protein concentration. N and NS mRNAs were translated in a rabbit reticulocyte lysate either individually (lanes a and b) or together (lanes c through f), with the amount of N mRNA constant (equal to that in lane a) and the amount of NS mRNA varied from the amount in lane b to 1/2, 1/4, or 1/8 (lanes c through f, respectively) of the amount in lane b. Samples were analyzed by nondenaturing PAGE.

may represent a regular set of multimers: N_2NS, N_4NS_2, N_6NS_3, N_8NS_4, $N_{10}NS_5$ (complex 5 to 1, respectively). However, there was sufficient scatter in the data to allow for the species N_3NS_2, N_5NS_3, or N_5NS_2 within the set. Also, we cannot rule out the possibility that tight binding to unlabeled protein(s) in the rabbit reticulocyte lysate determined the mobility of one or more of complexes 1 through 5.

Requirement of cotranslation for N-NS complex formation. The formation of N-NS complexes 1 through 6 was highly dependent on the simultaneous translation of N and NS proteins in the rabbit reticulocyte lysate. When N and NS proteins were translated individually and then mixed, under various conditions, there was little resultant complex formation (Fig. 3). Individually translated N and NS proteins (Fig. 3, lanes a and b) were incubated together for 30, 60, or 90 min at either 0 or 30°C prior to gel analysis (Fig. 3, lanes d to i). At either temperature, this treatment resulted in the formation of only a small amount of N-NS complex 6 and barely detectable amounts of complexes 1 through 5 (compared with cotranslated N and NS; Fig. 3, lane c), and the amounts of these complexes did not increase with time. Virtually all of each protein remained at the positions of the unmixed controls. This result suggested that either N or NS protein underwent some irreversible change shortly after its translation, which then prevented it from associating with the other protein. Since NS protein is phosphorylated at multiple sites in a manner that may affect the conformation and function of this molecule (19, 24), mixing experiments were also carried out in the presence of either ATP or calf intestinal alkaline phosphatase. However, neither of these conditions increased the amount of complex formation (data not shown). Thus, NS phosphorylation or dephosphorylation did not grossly influence N-NS complex formation; it may be that N protein was the limiting component in complex formation. This hypothesis is consistent with the demonstration by others that nascent N protein rapidly loses its ability to support in vitro replication of the VSV genome but can be maintained in a replication-competent state by cotranslated NS protein (17, 28). In the following report (26), we show that when N protein was translated alone in the rabbit reticulocyte lysate, it was tightly bound to RNA. This binding may have constituted an irreversible modification that rendered the protein unable to form N-NS complexes 1 through 6.

Dependence of relative amounts of N-NS complexes 1 through 6 on NS/N ratio. The spectrum of N-NS complexes formed upon cotranslation of N and NS was highly sensitive to the relative concentrations of the two proteins. This sensitivity, in turn, depended on the relative translation efficiencies of the N and NS mRNAs, which varied with the endogenous potassium and magnesium ion concentrations from lot to lot of rabbit reticulocyte lysate. Thus, the conditions favoring the formation of a particular subset of complexes were best determined empirically by performing cotranslation reactions in which the titers of a series of twofold dilutions of NS mRNA against a constant amount of N mRNA were determined (Fig. 4). A low NS mRNA/N mRNA ratio (1/8) left most of the N protein at its original position and mainly gave rise to N-NS complexes 1 through 3 (Fig. 4, lane f). An increase of the mRNA ratio to 1/4 allowed the use of more N protein and the production of roughly equal amounts of N-NS complexes 3 through 6, with a concomitant decline in the amounts of complexes 1 and 2 (Fig. 4, lane e). Further increase of the NS mRNA concentration resulted predominantly (Fig. 4, lane d) or exclusively (Fig. 4, lane c) in the formation of N-NS complex 6, with no

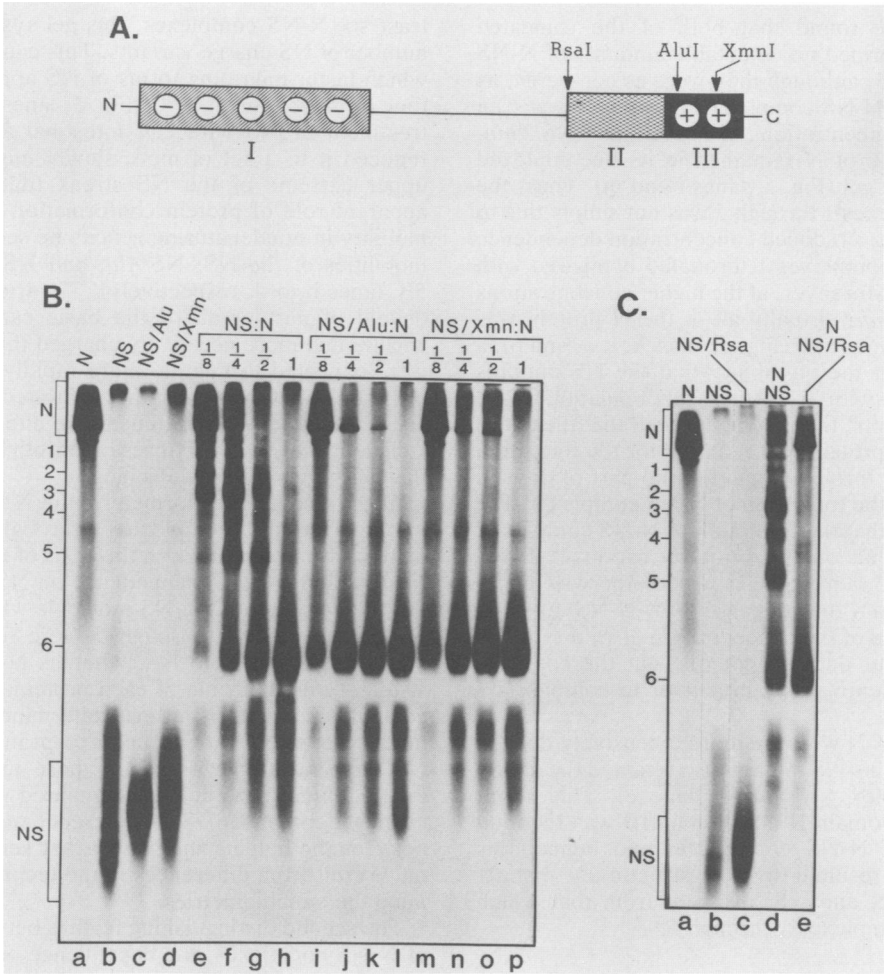

FIG. 5. Ability of NS protein mutants to form N-NS complexes 1 through 6. (A) Schematic diagram of the NS protein showing the functional domains I, II, and III, as defined by Gill et al. (14). Arrows denote the carboxy termini of NS protein deletion mutants obtained by translation of truncated mRNAs produced by runoff transcription of pGEM-NS2 which had been digested with *Rsa*I, *Alu*I, or *Xmn*I. The acidic (−) and basic (+) domains within the NS polypeptide are shown (14). (B) Formation of N-NS complexes 1 through 6 as a function of wild-type or mutant NS protein concentration. N and NS mRNAs were translated in a rabbit reticulocyte lysate either individually (lanes a through d) or together in N-NS pairs (lanes e through p), with the amount of N mRNA constant and the amount of NS mRNA (lanes e through h), NS/*Alu* mRNA (lanes i through l) or NS/*Xmn* mRNA (lanes m through p) varied as described in the legend to Fig. 4. (C) Ability of NS/*Rsa* mutant protein to form N-NS complex 6. N, NS, and NS/*Rsa* mRNAs were translated either singly (lanes a through c) or together in N-NS pairs (lanes d and e). The samples for panels B and C were analyzed by nondenaturing PAGE.

N protein left at its original position. This modulation of the type of complex formed by the relative concentrations of its components may have important regulatory implications. In the light of this variation, it should be noted that the determination of N-NS stoichiometries (Table 1) and the mixing experiments discussed above (Fig. 3) were performed at several NS/N protein ratios.

Ability of NS protein mutants to form N-NS complexes 1 through 6. Previous studies of the NS protein have defined three functional domains of this molecule (Fig. 5A). Domain I, consisting of the amino-terminal half of NS, accounts for the major part of the marked acidity of the molecule, because it is rich in glutamate and aspartate residues (13) and contains, in addition, numerous serines and threonines that serve as phosphorylation targets (5, 18, 23). Domain II, which spans amino acids 213 through 247 of NS (for VSV, New Jersey serotype), is a region, defined by deletion mapping (14), which interacts with the viral L protein and is essential for transcription (7, 14). Domain III, consisting of the carboxy-terminal 27 amino acids of NS, is the most basic portion of the molecule and is the region most conserved among the otherwise quite divergent phosphoproteins of the different VSV serotypes (13, 25). The removal of all (11, 14) or even just the 11 carboxy-terminal residues of domain III (D. Chattopadhyay, unpublished results) eliminates the ability of NS to bind to the viral N-RNA template in the absence of L protein but has no significant effect on the ability of NS to support transcription in vitro.

The potential contributions of NS domains II and III to the formation of N-NS complexes 1 through 6 were examined by the use of deletion mutants of NS. These mutants were generated by the translation of truncated mRNAs produced by runoff transcription of the vector pGEM-NS2 which had been digested with either *Alu*I, *Xmn*I, or *Rsa*I (14). The mutant proteins NS/*Alu* and NS/*Xmn* had the final 21 and the final 11 amino acids of domain III deleted, respectively (Fig. 5A). The mutant NS/*Rsa* had both domains II and III entirely deleted.

Wild-type NS, NS/*Alu*, and NS/*Xmn* were cotranslated with N protein, each over a range of NS concentrations (Fig.

5B). Surprisingly, we found that both of the truncated domain III mutants formed no detectable amounts of N-NS complexes 1 through 5, although they were as competent as wild-type NS to form N-NS complex 6. This result was seen even at the lowest concentrations of NS/*Alu* and NS/*Xmn*, for which the majority of N remained in its uncomplexed form at the top of the gel (Fig. 5, lanes i and m). Thus, the failure to form complexes 1 through 5 was not simply due to the NS mutants having a reduced concentration dependence for the formation of complexes 1 through 5 compared with that of wild-type NS. Moreover, at the higher concentrations of NS/*Alu* and NS/*Xmn*, virtually all of the N protein was converted to N-NS complex 6 (Fig. 5, lanes k, l, o, and p), a result which ruled out the possibility that the NS mutants could only combine with a certain subpopulation of N molecules. These results, then, demonstrated that the entire domain III of the NS protein was required for the formation of N-NS complexes 1 through 5 and that no part of domain III was necessary for the formation of N-NS complex 6. The results also indicated that the formation of N-NS complex 6, in fact, did not depend on the prior formation of N-NS complexes 1 through 5, a hypothesis which otherwise might have been inferred from the progression of N-NS complex formation as a function of the concentration of wild-type NS (Fig. 4). However, our data do not rule out the converse possibility that complex 6 was a precursor to complexes 1 through 5.

The cotranslation of N with the more extensively deleted NS/*Rsa* showed that this NS mutant also retained the ability to form N-NS complex 6 (Fig. 5C, lane e). This result showed that neither domain II nor domain III was required for the formation of N-NS complex 6, and hence, this complex must have resulted from a structurally distinct interaction between N and NS, different from that which gave rise to N-NS complexes 1 through 5.

DISCUSSION

It has previously been shown by Peluso and Moyer (30, 31) that in extracts prepared from VSV-infected cells, the majority of free N and NS proteins are specifically associated with each other. Davis et al. (8) investigated this association by using an in vitro RNA replication system containing a reticulocyte lysate programmed with purified N and NS mRNAs. These workers found that cotranslated N and NS proteins form complexes that can be partially resolved as one or two peaks by sedimentation through glycerol gradients. Since the stoichiometry of NS to N varies in progressive fractions across the gradient peaks, it was concluded that N and NS must form multiple complexes.

In this work, we have examined the complexes formed between the N and the NS proteins by producing wild-type or mutant mRNAs with the appropriate SP6 transcription vectors and translating the mRNAs in a rabbit reticulocyte lysate. Nondenaturing PAGE has proven to be an invaluable technique for analyzing the interactions between these two proteins, since we have been able to resolve at least six discrete N-NS complexes by this method. The mobility of a given molecule or complex of molecules in nondenaturing PAGE is a function of at least three parameters: charge, mass, and shape (or conformation). Thus, the smaller, more highly negatively charged NS molecule migrated much farther into the running gel than did the larger, less negatively charged N molecule (or N-RNA complexes [26]), and the various N-NS complexes exhibited mobilities intermediate between these two extremes. Besides clearly separating at least six N-NS complexes, this gel system also resolved a number of NS charge variants. This can be seen in Fig. 3, in which faster-migrating forms of NS appear as a function of time of incubation at 30°C (Fig. 3, lanes g to i). Conversely, treatment of NS with calf intestinal alkaline phosphatase reduced it to a set of more slowly migrating bands at the upper extreme of the NS streak (data not shown). The apparent role of protein conformation as a determinant of mobility in nondenaturing gel can be seen by comparing the mobilities of the NS, NS/*Alu*, and NS/*Xmn* proteins (Fig. 5B, lanes b to d, respectively). The truncated NS mutants, devoid of part or all of the basic carboxy terminus, are smaller and more negatively charged than wild-type NS and were expected to migrate more rapidly than the full-length protein; in fact, however, they migrated more slowly. Thus, small truncations of NS must have altered its conformation sufficiently to retard its passage through the gel, relative to that of the unaltered molecule.

The six complexes formed by the N and NS proteins fell into two groups, complexes 1 through 5 and complex 6, distinct from each other on the basis of three characteristics: (i) stoichiometry, (ii) dependence on NS concentration, and (iii) the domains on the NS molecule which interact with N. N-NS complexes 1 through 5 were found to have a 2:1 stoichiometry of N to NS, whereas N-NS complex 6 consisted of one molecule of each protein. These values are in general agreement with those determined by Davis et al. (8) in glycerol gradient fractions, except in the extreme of high NS concentrations for which these investigators found a limiting N/NS ratio of 0.67, compared with our value of 1.0 (Table 1; complex 6). This difference may reflect a variation between the Indiana and New Jersey serotypes of VSV, or it may result from differences in the techniques used to determine the stoichiometries.

The second distinguishing feature between the two groups of N-NS complexes, the dependence on NS concentration, was also noted by Davis et al. (8) to determine whether cotranslated N and NS will sediment as one or two peaks in glycerol gradients. In our present work, N-NS complexes 1 through 5 predominated at low concentrations of NS relative to N. On the other hand, N-NS complex 6 was the major or sole complex formed when NS was equimolar to or in excess of N (Fig. 4 and 5B). This concentration dependence is noteworthy in the light of in vitro VSV genome replication experiments by Howard et al. (17), which showed that the ability of cotranslated NS protein to maintain nascent N protein in a replication-competent state varies with the concentration of NS relative to N. Significantly, these workers found that replication is optimal at a 2:1 molar ratio of N to NS and declines as much as fourfold as the NS concentration increases. This result could imply that N-NS complexes 1 through 5 are the functional species involved in genome replication and that complex 6 represents a sequestered form of N. However, at variance with this notion, a recent report by Peluso and Moyer (31) suggests that a 1:1 molar N-NS complex, probably equivalent to our complex 6, is the active species involved in VSV genome replication.

Finally, we have demonstrated that the formation of N-NS complexes 1 through 5 required a fully intact domain III, the basic carboxy terminus of the NS protein (Fig. 5). In this respect, the interaction between N and NS in complexes 1 through 5 resembled the binding of NS to the viral N-RNA template, which has also been shown to be absolutely dependent upon domain III of NS (11, 14). In contrast, the formation of N-NS complex 6 was totally unaffected by the complete truncation of the entire carboxy-terminal regions

of the NS molecule, domains II and III. Thus, the interacting portion of the NS molecule in this complex lay elsewhere, perhaps in the highly acidic, multiply phosphorylated domain I (20). This finding of two classes of N-NS complexes is at variance with a report by Emerson and Schubert (11) who found that the binding of NS to viral N-RNA template is indistinguishable from NS binding to soluble N and that both require the carboxy terminus of NS. However, their conclusion was based on the examination of detergent-washed immune precipitates of mixed samples of separately translated N and NS. Our results clearly show that mixing of N and NS, rather than cotranslation, allowed only a very small fraction of each to form N-NS complexes (Fig. 3); moreover, nonionic detergents have been shown to disrupt N-NS complex formation (8).

It is striking that the two separate types of binding of NS to N were analogous to the two types of binding exhibited by two monoclonal antibodies to N described by Arnheiter et al. (1). Antibody 1 binds the nucleocapsid N-RNA and the free N protein (similar to NS in complexes 1 through 5), whereas antibody 2 binds only to free N protein (similar to NS in complex 6). It is conceivable that these two monoclonal antibodies can differentiate between the two groups of N-NS complexes. Indeed, Davis et al. (8) found, under conditions in which we would now expect N-NS complex 6 to be the predominant product, that antibody 1 binds efficiently to N-NS complexes, but antibody 2 fails to do so. Thus it would appear that in complex 6, NS and antibody 2 compete for the same domain(s) of the N protein.

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